Expression of Interleukin-18 and Caspase-1 in Cutaneous T-Cell Lymphoma

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Abstract Purpose: Cutaneous T-cell lymphoma (CTCL) is a malignancy of skin-homing Th2 T cells. Clonal T cells and CTCL skin lesions typically express Th2 cytokines, including interleukin (IL)-4, IL-5, and IL-10, but fail to produce Th1 cytokines. However, the reason for Th2 bias is unknown. IL-18 is a pleiotropic proinflammatory cytokine produced by monocytes/macrophages lineage as well as epithelial cells, such as human keratinocytes. In the absence of IL-12, IL-18 leads to increased immunoglobulin E production from B cells and enhanced production of IL-4 and IL-13 by basophils, mast cells, and CD4+ T cells. We have analyzed cytokines in CTCL patients, which may bias the immune response around the Th1/Th2 axis.

Experimental Design: We examined plasma of 95 CTCL patients and skin of 20 CTCL patients for IL-18, caspase-1, IL-12, and other cytokines. To identify the presence or absence of these cytokine proteins in CTCL and normal skin, we cultured explants from skin biopsies on three-dimensional matrices.

Results: Plasma levels of IL-18 and its converting enzyme, caspase-1, were significantly elevated in CTCL. mRNA levels for these factors were also elevated in CTCL skin lesions. Matrices populated with CTCL lesionalskin produced significant amounts of IL-18 and caspase-1; however, production of IL-12 protein was barely detectable.

Conclusions: We propose that the high levels of IL-18 expression in lesional CTCL skin contribute to increased plasma levels of IL-18 and that this, in the face of significantly lower levels of IL-12, may contribute to the Th2 bias seen in this disease.

Cutaneous T-cell lymphoma (CTCL) encompasses a spectrum of lymphoproliferative disorders of the skin (1). They represent a subset of extranodal non-Hodgkin’s T-cell lymphomas and are increasingly recognized to be malignancies of skin-homing Th2 T cells (2). These T cells may be found singly or in collections within the epidermis and are accompanied by dermal infiltrates of nonmalignant T cells and other mononuclear cells. These infiltrating cells as well as resident cells, such as keratinocytes and fibroblasts, produce a variety of cytokines in situ, which modulate cutaneous inflammation (3).

A prominent feature of CTCL is immunosuppression, and many CTCL patients, especially those in advanced stages, are at significant risk of succumbing to bacterial and viral infections (4). The etiology of this immunosuppression is unknown and likely to be multifactorial. We reported previously that the T-cell repertoire in CTCL patients is significantly contracted (5) despite the presence of relatively normal absolute numbers of T cells (6, 7). Furthermore, the levels of T-cell receptor excision circles in patients with CTCL were significantly decreased even in patients with early-stage disease (8). The contraction of the T-cell repertoire very likely contributes to the immune suppression and significant infection-related mortality that characterizes advanced CTCL. In addition to these abnormalities, clonal T-cell populations in CTCL produce Th2 cytokines (9–11) and this may be associated with reduced T-cell-mediated cellular immune responses and diminished natural killer cell activity (12) that are also identified as features of the immunosuppression of this disease.

The basis of the Th2 skewing in CTCL is presently unknown, and the present study was designed to test the hypothesis that cytokines released within lesional skin may contribute to this process. Interleukin (IL)-18 is a pleiotropic proinflammatory cytokine produced primarily by monocytes/macrophages lineage, Kupffer cells, and dendritic cells as well as epithelial cells, such as human keratinocytes (13, 14). IL-18, like IL-1β, is synthesized as an inactive precursor (pro-IL-18, 24 kDa) and then cleaved by the IL-1β-converting enzyme, caspase-1, into
an active 18-kDa mature form and secreted. In combination with IL-12, the 18-kDa mature form can induce IFN-γ production by T cells (13, 15) and suppress immunoglobulin E (IgE) production from B cells. However, high levels of IL-18 alone lead to increased IgE levels and enhanced production of IL-4 and IL-13 by basophils, mast cells, and CD4+ T cells (16, 17). Thus, in the absence of IL-12, IL-18 seems to favor a Th2 response in vivo. Transgenic mice that overexpress IL-18 (18) or caspase-1 (19) in their keratinocytes showed high serum IL-18 and IgE levels. Moreover, normal mice that received skin grafts from caspase-1 transgenic donor mice showed long-lasting elevations of serum IgE levels that persisted after the grafted skin was removed (20). Thus, epidermal IL-18 can contribute to nonspecific Th2-type skewing and durable systemic IgE production.

Aberrant expression of IL-18 has been detected in certain inflammatory skin disorders. Patients with the common Th2 skin disease atopic dermatitis have high serum levels of IL-18 (21–23), and a study of five CTCL patients and one with cutaneous natural killer cell lymphoma also showed higher serum levels of IL-18 than normal controls (24). However, the origin and the role of IL-18 in these diseases have not been fully determined. In the present study, plasma samples from 95 CTCL patients in all stages of disease were analyzed for the presence of IL-1β, IL-2, IL-4, IL-12, IL-13, IL-18, and caspase-1. In parallel, skin biopsies from some of these patients and controls were analyzed for expression of cytokine and caspase-1 mRNA. Furthermore, additional skin biopsies were cultured and assayed for the production of cytokine and caspase-1 protein.

Our results suggest that plasma levels of IL-18 and caspase-1 are indeed elevated in CTCL patients regardless of stage and that IL-18 and caspase-1 mRNA and protein are both overexpressed in lesional skin. Whereas IL-12 p40 and IL-23 p19 mRNAs were overexpressed in CTCL skin, IL-2p35 mRNA was not, and IL-12 protein was barely detectable (<3 pg/mL). These results are consistent with the idea that IL-18 from lesional CTCL skin, in the absence of IL-12, may contribute to Th2 skewing in this disease.

Materials and Methods

Patients and healthy donors. After their informed consent, patients with CTCL were recruited for this study from the Cutaneous Oncology Clinic at the Dana-Farber Cancer Institute. CTCL was classified and staged according to the WHO primary tumor, regional nodes, and metastasis classification. A total of 95 CTCL patients (53 men and 42 women; median age, 61 years; range, 19-94 years) were recruited for this study. The subject profiles are as follows: stage I (37 men and 28 women; median age, 60 years; range, 19-90 years), stage II (6 men and 2 women; median age, 67 years; range, 31-82 years), stage III (8 men and 7 women; median age, 63 years; range, 30-94 years), and stage IV (2 men and 5 women; median age, 65 years; range, 57-78 years). Diagnoses were based on clinical criteria as well as histologic and immunohistologic assessment of skin specimens. Blood specimens obtained from 20 healthy volunteers (10 male and 10 female; median age, 40 years; range, 24-53 years) were also studied for comparison.

Skin biopsy samples were taken from the lesions of 20 CTCL patients (12 men and 8 women; median age, 63 years; range, 29-94 years) under local lidocaine/epinephrine anesthesia after obtaining their informed consent. The subject profiles are as follows: stage I (8 men and 6 women), stage II (2 men and 1 woman), stage III (1 man), and stage IV (2 women). None of the patients had received any UV treatment, systemic drug therapy, or topical corticosteroids for at least 3 weeks before the investigation. Ten normal human skin samples were obtained as discarded tissue from cutaneous surgeries and were divided into 6 x 6–mm portions, the same size as skin biopsy specimens from CTCL patients. All studies using blood and skin biopsy samples were approved by the Dana-Farber Cancer Institute Institutional Review Board under protocol 2016 entitled “Collections of tissue and blood specimens and clinical data from patients with cutaneous T-cell lymphoma.”

Plasma preparation. Plasma samples were isolated from heparinized venous blood with density gradient centrifugation over Ficoll-Histopaque (Sigma, St. Louis, MO). All plasma samples were stored at −80°C before use.

Quantification of cytokines. Cytokine levels for plasma and skin culture supernatants were measured by ELISA. The IL-18 ELISA, which detects only the 18-kDa mature form, was purchased from MBL Co. (Nagoya, Japan). ELISAs to detect caspase-1, IL-1β, IL-2, IL-4, IL-12, IL-13, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The IgE ELISA was from IBL (Hamburg, Germany). Samples were thawed at room temperature and assayed in duplicate. The reproducibility of the ELISA was assessed through the incorporation of a control plasma sample in each assay.

Quantitative measurement of cytokine mRNA levels. For quantitative reverse transcription-PCR analysis, biopsy specimens taken from 10 CTCL patients and 10 normal donors were snap frozen in liquid nitrogen until use. After homogenization of the skin biopsy specimen, total RNA was extracted using the RNA purification kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Total RNA (2-5 μg: A 260/A 280 = 1.7-2.0) was reverse transcribed with oligo(dT) primers and Powerscript reverse transcriptase (Clontech) in a final volume of 20 μL. Quantitative PCR reactions were done using an iCycler machine (Bio-Rad, Hercules, CA) using 1 μL cDNA in a final volume of 25 μL with SYBR Green PCR Core Reagents (Biosystems, Warrington, United Kingdom) and 200 nmol/L of the specific primers. Samples were screened for the expression of β-actin as a reference gene. The primer pairs specific for IL-18, caspase-1, IL-1β p35 and p40 subunits of IL-12, p19 subunit of IL-23, IFN-γ, and β-actin were as follows: IL-18, 5'-CATACAACTTCCATGGGAAGTTAATCAA-3' and 5'-CATA-TGGATCCCCAATCTGCTG-3' (25); caspase-1, 5'-CAAGGTTGCTGAAACTG-3' and 5'-GGGCAATGGTGATGTTGTC-3' (25); IL-1β, 5'-AGTACCTGTCGCTCCAGTG-3' and 5'-CTGCTTGTTGCTGATG-3' (26); IL-2 p35 subunit, 5'-ACCCAGAATGTTTCATGTC-3' and 5'-TCCCTGACGAACGTCATGCTGTC-3' (27); IL-2 p19 subunit, 5'-ACTGCAATTCAGCGAATGTCATGCTGCAGAA-3' and 5'-TCCCTGACGAACGTCATGCTGTC-3'. A series of standard dilutions of a plasmid were used to quantify cytokines and enzyme. Specific signals for all transcripts were readily detected in cDNA prepared from neutrophils treated with 100 ng/mL purified tetanus toxoid (Massachusetts Biologic Laboratories, Worcester, MA) for 8 hours. Standard dilutions were amplified with pGEM-T Easy Vector Systems (Promega, Madison, WI) from the PCR amplifiers above. PCR was conducted with 40 cycles, which were within the linear amplification range for all PCR reactions. All PCR reactions for these samples were conducted twice. The specificity of the PCR products was confirmed by sequence analysis.

Preparation of three-dimensional skin explant cultures. To analyze skin-producing cytokines in lymphocyte-tissue interactions, we developed three-dimensional skin explant cultures (details are mentioned in ref. 29). Briefly, 9 x 9 x 1.5–mm Cellfoam matrices (Cytomatrix,
Woburn, MA) were autoclaved and incubated in a solution of 100 μg/mL rat tail collagen I (BD Biosciences, Bedford, MA) in PBS for 30 minutes at 37°C. A punch biopsy was taken from lesional CTCL skin or from normal discard skin, s.c. fat was removed, and the tissue was minced into explants –2 × 2 × 2 mm. Three skin explants were placed on the surface of each matrix, and each matrix was placed in an individual well of a 24-well culture plate (16 mm in diameter). The matrices with explants were covered with 2 mL/well Iscove’s modified medium (Mediatech, Herndon, VA) with 10% heat-inactivated fetal bovine serum (Sigma), penicillin, streptomycin, and 3.5 μL/L β-mercaptoethanol. Half of the medium (1 mL) was removed and replaced with fresh medium thrice weekly. Cultures were maintained in this fashion for 3 weeks. In this system, keratinocytes and dermal fibroblasts emigrate and colonize the matrices. The matrices are also consistently found to become populated with T cells. Some normal skin explants were also treated with Th2 cytokines. Th2 cytokine treatment involved addition of 25 ng/mL IL-4 and IL-13 into the culture medium from day 1. Ten CTCL skin samples and 10 normal control samples were collected for this experiment.

Statistics. Linear regression models were fitted to the plasma IL-18 and caspase-1 level data from all patients. The models included logarithmic values of the plasma IL-18 or caspase-1 level data as the dependent variable and gender and stage as the independent variables. We also examined the correlations between log plasma IL-18 and caspase-1 levels. The Wilcoxon-Mann-Whitney test was used to evaluate differences in cytokine and enzyme mRNA expression levels between the normal skin and CTCL lesion skin samples and to compare actual cytokine production from CTCL and normal skin matrices.

Results

Plasma cytokine levels. Plasma cytokine levels were measured in 95 patients and 20 controls as described in Materials and Methods. ELISA data for plasma IL-12, IL-1β, IFN-γ, IL-4, IL-13, IL-2, and IgE did not show differences between any stage and normal controls that reached significance (data not shown). Of all cytokines examined, only IL-18 and caspase-1 were found to be elevated in patients with CTCL relative to normal controls. When the data describing plasma cytokine levels were log transformed, the data became symmetrical and were analyzed further. A linear regression model was fitted to the log10 of plasma IL-18 levels data with gender and stage as covariates. Although gender was not a significant covariate, significant differences were observed between stage I versus normal, stage II versus normal, stage III versus normal, and stage IV versus normal. A multiple comparison procedure was used to determine whether there were differences in IL-18 expression between the stages, and it was determined that there were no significant differences in IL-18 expression between any of the stages. Therefore, patients from all stages were grouped into a single population and compared with the group of healthy volunteers. The linear regression model was fitted again with the following covariates: gender and stage defined as normal versus CTCL. The results are shown in Fig. 1A and Table 1. The log10 of IL-18 levels differed significantly between normal volunteers and CTCL patients.

Levels of plasma caspase-1 were also measured in the same patient populations, and data were analyzed in a fashion similar to the IL-18 data. A linear regression model was also fitted to the log10 of plasma caspase-1 levels with gender and stage as covariates. The results were similar to those from the model that fitted plasma IL-18 levels (i.e., differences were observed between normal volunteers and patients with all stages of CTCL). Again, similar to IL-18, there were no significant differences in caspase-1 expression between the stages. Figure 1B and Table 2 present the results from the fitted linear regression model with gender and stage (CTCL versus normal) as covariates.

Next, a linear regression model was fitted to log plasma IL-18 levels with gender, stage, and caspase-1 levels as covariates (Fig. 2 and Table 3). The model was fitted to data from CTCL patients only and did not include data from the healthy volunteers. Plasma caspase-1 seems to be a significant covariate.

Table 1. Parameter estimates

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Fig. 1. Ninety-five CTCL and 20 normal plasma samples were analyzed with ELISA. IL-18 and caspase-1 were significantly elevated in CTCL. Bars, SE. *, P < 0.001; **, P < 0.01; ***, P < 0.05.
To further explore the association between log plasma IL-18 and plasma caspase-1, we looked at the Spearman correlation coefficient. The correlation coefficient between log plasma IL-18 and log plasma caspase-1 was 0.31 (Fig. 2), suggesting that these two variables were not independent and that expression of IL-18 and caspase-1 in plasma are related.

**Cytokine transcript expression in CTCL and normal skin.** mRNA expression levels for cytokine and caspase-1 in skin biopsies were measured by quantitative PCR analysis. The two-sided Wilcoxon-Mann-Whitney test was used to assess the differences in IL-18, caspase-1, IL-1β, IL-12 p35 and p40, IL-23 p19, and IFN-γ between 10 normal skin and 10 CTCL lesion skin samples. Figure 3 shows the results of quantitative PCR reactions for the cytokines. Significant differences between CTCL and normal skin were seen in IL-18 (P < 0.0001), caspase-1 (P < 0.0001), IL-1β (P < 0.0001), IL-12 p40 (P = 0.0115), IL-23 p19 (P = 0.0004), and IFN-γ (P = 0.0004). Although no differences were detected in the levels of mRNA encoding IL-12 p35, the mRNA levels of IL-18, caspase-1, IL-1β, IL-12 p40, IL-23 p19, and IFN-γ transcripts were significantly more highly expressed in CTCL skin lesions than in normal controls.

**Detection of cytokines and caspase-1 protein in supernatants from CTCL and normal skin explants.** Supernatants from three-dimensional skin explant culture systems were collected at week 3 and cytokine production was analyzed using ELISAs for IL-18, caspase-1, IL-1β, and IL-12 (no ELISA for IL-23 is currently available). The two-sided Wilcoxon-Mann-Whitney test was used to assess the differences in IL-18, caspase-1, IL-1β, and IL-12 between normal controls and CTCL patients. Significant differences between normal and CTCL skin were seen in IL-18 (P < 0.0001), caspase-1 (P < 0.0001), and IL-12 (P = 0.0011) levels (Fig. 4). IL-12 was undetectable in normal skin supernatants, and levels detected in CTCL, although statistically significant, were extremely low (<3 pg/mL). Neither IL-4 nor IL-13 affected the result for cytokine and caspase-1 levels (Fig. 4). No other cytokine proteins were expressed at significant levels in these cultures.

**Discussion**

In this study, we have shown that plasma levels of IL-18 are significantly elevated in patients with CTCL, thus extending the finding of a previously published small study to a large cohort of patients. Interestingly, there was no trend toward higher levels of IL-18 as the stage of CTCL increased. In parallel, caspase-1, which cleaves the biologically inactive precursor of IL-18 into a mature active protein, was also elevated in CTCL, and this elevation was again independent of stage. However, levels of IL-18 and caspase-1 were interdependent and tended to increase in parallel according to linear regression analysis. This observation is, to our knowledge, novel and has not been reported previously.

We next asked whether lesional skin might be the source of the elevated plasma levels of IL-18 and caspase-1. Quantitative PCR using mRNA taken from normal control skin revealed low but detectable levels of IL-18 and caspase-1 mRNA expression. This finding is consistent with previous studies showing that human keratinocytes are capable of synthesizing low levels of IL-18 and caspase-1 mRNA even under nonstimulated conditions (30–32). In experiments not shown here, cultured normal keratinocytes, rather than fibroblasts, seem to be the dominant source of IL-18 and caspase-1. In lesional CTCL skin, IL-18 and caspase-1 mRNA were clearly elevated compared with normal skin.

To further study the origins of these cytokines, we used a novel organ culture system. Explants of human normal and CTCL skin were placed on three-dimensional tantalum-coated carbon matrices (Cellfoam). The matrices were colonized by resident skin cells, including keratinocytes, fibroblasts, and skin resident T cells, and provide an environment allowing for the maintenance of lesional T cells without their proliferation in situ. We collected and analyzed the supernatants from these cultures. Supernatants of CTCL skin explants showed significantly elevated levels of mature IL-18 as well as caspase-1 (Fig. 4A and B). It has been reported that IL-18 gene expression may be up-regulated via activation of Toll-like receptors after stimulation with microbial products, such as lipopolysaccharide, or by cytokines, such as IFN-α and IFN-γ (33–35). We cannot exclude the possibility that CTCL skin may be colonized with bacteria that may contribute to IL-18 production. Some of our patients received IFN-α; however,
no patients with stage I disease were given this drug, whereas plasma IL-18 levels are elevated even in stage I. We believe that elevated IL-18 levels in CTCL patients are induced primarily by the interaction between skin-infiltrating T cells, keratinocytes, and fibroblasts. We also looked for the expression of IL-1β, another caspase-1-dependent cytokine, and IL-12 using sensitive ELISA assays. Levels of both cytokines were detectable in supernatants of CTCL explants, although at levels lower than 3 pg/mL. IL-12 was undetectable in supernatants of normal skin explants (Fig. 4A).

Clonal T cells in CTCL peripheral blood produce Th2 cytokines (9–11) and CTCL is considered to be a Th2 disease. As noted previously, IL-18 is known to increase circulating levels of IgE. Indeed, in several CTCL patients, we detected high levels of plasma IgE by ELISA (data not shown). However, differences in plasma IgE levels between normal controls and various stages of CTCL did not reach statistical significance, contrary to previous reports (36), nor did plasma IgE levels correlate with plasma IL-18 or caspase-1 levels. It is worth noting that in our sample many of the CTCL patients with low plasma IgE levels have been treated previously with IFN-α, raising the possibility of a causal relationship.

Another striking result of our study is the relative absence of IL-12 expression in CTCL skin. IL-12 is a heterodimeric molecule consisting of disulfide-linked 35- and 40-kDa chains and is secreted by a variety of cells, including macrophages and B cells. Low levels of both IL-12 p40 and p35 transcripts have been detected in keratinocyte cultures (37). The biological activity of IL-12 is confined to the p70 heterodimer, and simultaneous expression of the two genes encoding IL-12

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Fig. 3. IL-18, caspase-1, IL-1β, IL-12, IL-23 p19, and IFN-γ mRNA levels from skin biopsy. Skin biopsy samples were taken from the lesions of 10 CTCL patients and 10 normal control subjects. mRNA expressions were analyzed with quantitative PCR. Bars, SE. *P < 0.001; **, P < 0.05.
p35 and p40 chains is required for its production (38). We investigated expression of IL-12 p35 and p40 mRNA in normal and CTCL skin. IL-12 p35 transcript levels were low and did not differ significantly across all samples (Fig. 3D). This ubiquitous low expression of the IL-12 p35 subunit is in accordance with published data showing constitutive p35 expression in keratinocytes (37). In contrast to the uniform low p35 subunit levels, IL-12 p40 mRNA signals were significantly higher in CTCL skin than in normal skin (Fig. 3E). IL-12 production from CTCL skin and normal controls was also analyzed with ELISA. Although differences were significant, concentrations were very low (below the limit of detection in normal explant cultures and 1.7 pg/mL in CTCL explant cultures; Fig. 4A). In CTCL lesions, both keratinocytes and skin-infiltrating mononuclear cells may produce biologically active IL-12 p70 heterodimer. However, our findings indicate that the concentration of IL-12 produced in lesional skin is orders of magnitude lower than that of IL-18. IL-12 and IL-18 synergize to promote the development of Th1 cells (39–41). However, in the absence of elevated IL-12, IL-18 supports the production of Th2 cells (16, 17). Thus, the levels of IL-12 in CTCL lesions, although detectable, may be insufficient to redirect the effects of IL-18 toward Th1 development. Any propensity for Th1 differentiation caused by very low levels of lesional IL-12 seems to be strongly outweighed by the Th2 skewing effect of high levels of IL-18 expression.

The more recently discovered cytokine IL-23 consists of a heterodimer of the IL-12 p40 subunit and a novel 19-kDa protein, termed p19. p19 only becomes biologically active when complexed with p40 (42). IL-23 is now recognized to play a role in the recruitment of inflammatory cells in Th1-mediated diseases. In psoriasis patients, p19 was found to be more highly expressed in lesional skin than in nonlesional skin (43). We found that IL-23 p19 mRNA was overexpressed in CTCL lesions (Fig. 3F). The significance of this is unknown, partly because confirmation of protein production was impossible in the absence of an ELISA for IL-23.

Finally, another interesting function of IL-18 is up-regulation of cytotoxic activities of natural killer cells and cytotoxic T cells (35, 44–47). Although IL-18 and IL-12 exert the induction of IFN-γ synergistically, IL-18 up-regulates cytotoxic activities of effector cells independently of IL-12. These effector cells seem to be profoundly involved in the development of inflammatory immune responses. CTCL patients with higher levels of CD8+ cells in their lesional skin tend to have less aggressive clinical courses, suggesting that an antitumor immune response is important in this lymphoma.

In conclusion, our data show enhanced production of the cytokine IL-18 and of the enzyme caspase-1 in CTCL plasma and skin compared with normal plasma and skin. The skin-produced cytokine and enzyme have the potential to significantly influence immune status. The up-regulation of IL-18 can initiate and promote a Th2 cytokine profile, thus contributing to the Th2 inflammatory response in CTCL. Although Th1 skewing cytokines, such as IL-12 and IFN-γ, are also present in CTCL skin lesions, their levels are at the lower limits of detection, and their effects do not seem to be sufficient to skew the CTCL milieu toward Th1.

Many CTCL patients show signs of impaired cellular immunity, including elevated mortality from bacterial infections (4). Th2 skewing, mediated by increased IL-18 levels, may play an important role in this immunosuppression, especially in advanced stages. Therapeutic approaches supporting Th1-type immune response using IFN-α (48), IFN-γ (49), IL-12 (50), and adenovirus-mediated IFN-γ gene transfection (51) have been attempted, with various degrees of success. However, our findings raise the possibility that neutralization of IL-18 with the goal of reversing this pathogenic Th2 bias may be a promising therapeutic approach to reversing the immunosuppression associated with CTCL.

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References


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